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(72) Inventeurs/Inventors:
LANGFORD, KELLY, GB;
BURDON, DREW, GB;
WHYTE, MUNA, GB

(73) Propriétaire/Owner:
SMITH & NEPHEW PLC, GB

(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : FORMATION D'UN SUPPORT A PARTIR DE PLUSIEURS COMPOSANTES DE SUPPORT PLUS PETITES
(54) Title: FORMATION OF A SCAFFOLD FROM MULTIPLE SMALLER SCAFFOLD COMPONENTS

(57) Abrégé/Abstract:
The invention relates to methods of preparing tissue implants for use in the augmentation, repair and regeneration of tissues. The method of preparing such tissue implant is carried out in vitro, and involves providing cells isolated from a tissue source; seeding at least two primary scaffolds with the cells and culturing the at least two primary scaffolds for a period of time sufficient for the cells to secrete an extracellular matrix; and subsequently loading the at least two primary scaffolds obtained into a secondary scaffold, whereby the secondary scaffold contains and protects the at least two primary scaffolds.

Abstract

The invention relates to methods of preparing tissue implants for use in the augmentation, repair and regeneration of tissues. The method of preparing such tissue implant is carried out *in vitro*, and involves providing cells isolated from a tissue source; seeding at least two primary scaffolds with the cells and culturing the at least two primary scaffolds for a period of time sufficient for the cells to secrete an extracellular matrix; and subsequently loading the at least two primary scaffolds obtained into a secondary scaffold, whereby the secondary scaffold contains and protects the at least two primary scaffolds.

FORMATION OF A SCAFFOLD FROM MULTIPLE SMALLER SCAFFOLD COMPONENTS

FIELD OF THE INVENTION

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The present invention finds applicability in the field of tissue culture as well as in the field of preparing tissue substitutes for tissue replacement.

10 ## BACKGROUND

Articular cartilage consists of highly specialised chondrocytes surrounded by a dense extracellular matrix consisting mainly of type II collagen, proteoglycan and water. This avascular tissue has a
15 limited ability of repair. Damage of cartilage produced by disease, such as rheumatoid arthritis and/or arthritis, or trauma can lead to serious physical deformity and debilitation.

Previous tissue engineering solutions for cartilage repair have
20 focused around the production of a continuous piece of cartilage capable of repairing a cartilage defect in its entirety. This approach has significant drawbacks.

The size of the piece of cartilage which can be produced *in vitro* is
25 limited. Large scale cell-scaffold cultures have limitations in terms of controlling proliferation and differentiation. These problems are primarily caused by insufficient nutrient diffusion through the scaffold, causing cell necrosis and areas of undifferentiated cells within the scaffold.

30

A further problem is that the dimensions of cartilage defects vary from patient to patient. As a consequence, pieces of cartilage have to be tailored to an implant site, multiple pieces of cartilage have to be tessellated in order to repair a large defect or the technique
35 referred to as 'mosaicplasty' is used which requires the defect to be

prepared to enable a standard sized cartilage replacement implant to be inserted.

The use of cell microcarriers for fabricating cell-containing implants
5 has addressed some of these issues. Typically cells are seeded onto resorbable microcarriers, such as spherical beads, cubes, cylinders or plates at low density and then culture-expanded to form aggregates. These cell micro-carrier aggregates can then be formulated as an injectable dispersion of aggregates or as a solid
10 structure of consolidated aggregates prepared by further culturing of aggregates in a mold device having a geometry reflecting that of the defect. However a significant drawback with this technique is the reliance on the adherence of the cells to the micro-carrier surface. It is often necessary to coat the surface with, for instance, a bioactive
15 peptide in order to improve the adhesion. Additionally, because there is often miminal resorption of the microcarrier during the culture expansion step, with significant rates of resorption only occurring once the microcarrier is implanted, this can have a significant influence on the healing process.

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SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided a method of preparing a tissue implant *in vitro*, said method comprising:

25 (a) providing cells isolated from a tissue source;

(b) seeding at least two primary scaffolds with the cells and culturing the at least two primary scaffolds for a period of time sufficient for the cells to secrete an extracellular matrix; and

30 (c) loading the at least two primary scaffolds obtained in (b) into a secondary scaffold whereby the secondary scaffold contains and protects the at least two primary scaffolds.

According to a second aspect of the invention there is provided a method of augmenting, repairing or regenerating tissue within a subject, said method comprising the steps of:

5 (a) obtaining cells from a suitable tissue source;
(b) seeding at least two primary scaffolds with the cells and culturing the primary scaffolds for a period of time sufficient for the cells to secrete an extracellular matrix;
(c) loading the at least two scaffolds obtained in (b) into a
10 secondary scaffold and;
(d) implanting the secondary scaffold into a site within the subject.

The extracellular matrix producing cells are seeded onto the primary
15 scaffolds which are small 3-D scaffolds composed preferably of non-woven fibres of a biocompatible, biodegradable material.

An example of a suitably sized scaffold has a diameter of about 1-3mm and a depth of about 0.5-3mm, more particularly a diameter of
20 about 1-2mm and a depth of about 0.5-1mm.

The primary scaffolds can be of any suitable shape, for a cylinder, a disc or a cube.

25 The cells are seeded, preferably uniformly, onto a primary scaffold with typically between about 50,000 and $1x10^6$ cells per scaffold, or between about 100,000 and 300,000 cells per scaffold or more particularly between about 150,000 and 250,000 cells per scaffold.

30 A typical seeding density on a scaffold is between about 100,000 and 3×10^6 cells/mm^3, or between about 300,000 and 800,000 cells/mm^3 or more particularly between about 400,000 and 700,000 cells/mm^3.

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In specific embodiments of the invention the typical seeding density of cells on a primary scaffold is about 650,000 cells/mm^3.

The cells may be seeded onto scaffolds using methods known to
5 those skilled in the art. These include, but are not limited to, pipetting a suspension of cells onto the scaffold, incubating the scaffold in a suspension of cells for long enough that a proportion of the cells adhere to at least part of the surface of the scaffold or by combining the scaffolds and cells in a liquid and centrifuging to facilitate contact
10 between the two components.

These cell-seeded scaffolds are then cultured for a period sufficient for the cells to produce cell aggregates. The primary scaffolds can be cultured in, for example, a conical flask, a roller bottle, a techne
15 flask or a bioreactor.

In embodiments of the invention the cells are cultured on the scaffold for at least 12 hours to ensure appropriate aggregate formation, with continuous flow in a low volume of media. This low volume of culture
20 media is relative to the number of scaffolds being cultured in any particular size of culture vessel and encourages aggregate formation by bringing the cell seeded scaffolds into close contact with each other. The volume of media is then increased and the cell-seeded scaffolds cultured under static conditions or with intermittent or
25 constant agitation. Following an appropriate incubation period the at least two cell-seeded primary scaffolds are loaded into a secondary scaffold. This secondary scaffold can then be directly implanted into the site within a subject or alternatively cultured prior to implantation.

30 The incubation of the secondary scaffold prior to implantation allows the primary scaffolds to fuse into a larger aggregate which may be more stable at the time of implantation. These scaffolds are typically incubated for between 12 hours and 7 days.

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The use of a secondary scaffold to contain the primary scaffolds provides additional mechanical strength and protection for the primary scaffolds during implantation and additionally while the tissue is being regenerated or repaired.

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The second scaffold can be designed in various configurations, including but not limited to, cups, discs, cubes and cylinders.

The secondary scaffold may be reinforced to provide extra
10 mechanical strength, for example with glass or polymeric fibres.

A drawback associated with a number of the prior art scaffolds used as the basis for tissue implants, for example non-woven and woven felts, ceramics, sponges, is the addition of a level of manufacturing
15 complexity and cost into the production process. The use of fibres is predicted to reduce the complexity of manufacturing and therefore the costs. The use of fibres also allows for the development of more high-throughput seeding techniques.

20 Therefore according to a third aspect of the invention there is provided a method of preparing a tissue implant *in vitro*, said method comprising the steps of;

(a) providing cells isolated from a suitable tissue source,
25 (b) providing a plurality of fibres, and;
(c) culturing the cells and fibres together for a period of time sufficient for the cells to secrete an extracellular matrix and form a cellular aggregate.

30 According to a fourth aspect of the invention there is provided a method of augmenting, repairing or regenerating tissue within a subject, said method comprising the steps of:

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(a) obtaining cells from a suitable tissue source;
(b) providing a plurality of fibres,
(c) culturing the cells and fibres together for a period of time sufficient for the cells to secrete an extracellular matrix and form a cellular aggregate, and;
(d) implanting the aggregate into a site within the subject.

An example of a suitably sized fibre has a diameter of about 10-100μm and a length of about 0.5-5mm, more particularly a diameter of about 10-100μm and a length of about 0.5-3mm, even more preferably a diameter of about 10-100μm and a length of about 1-3mm.

If it is desired that the scaffolds are of a substantially cylindrical shape, then the diameter of the fibre is preferably half its length.

The cells can be mixed with the fibres using methods known to those skilled in the art. These include, but are not limited to, pipetting a suspension of cells onto the fibres, incubating the fibres in a suspension of cells for long enough that a proportion of the cells adhere to at least part of the surface of the fibres or by combining the fibres and cells in a liquid and centrifuging to facilitate contact between the two components.

This cell/fibre mixture is then cultured for a period sufficient for the cells to secrete an extracellular matrix and form cellular aggregates. Culturing can take place in, for example, a conical flask, a roller bottle, a techne flask or a bioreactor.

In embodiments of the invention the cells are cultured with the fibres for at least 12 hours to ensure appropriate aggregate formation, with continuous-flow in a low volume of media. This low volume of culture media is relative to the number of fibres being cultured in any

particular size of culture vessel and encourages aggregate formation by bringing the cells and fibres into close contact with each other. The volume of media is then increased and the cell-fibre aggregates are cultured under static conditions or with intermittent or constant
5 agitation.

It is preferable that the fibrous material has completely resorbed by the end of the culture period or that only a residual amount of fibrous material remains, with the fibrous material having been replaced by
10 secreted extracellular matrix.

The fibres function as a scaffold for the cells during the aggregation of the cells.

15 A typical cell seeding density is from about 2 $x10^4$ to about 3 $x10^6$ cells/μg of fibre, or from 3 $x10^4$ about to about 3 $x10^5$ cells/μg of fibre or more particularly from about 4 $x10^4$ to about 7 $x10^4$ cells/μg of fibre.

20 In specific embodiments of the invention the typical seeding density is about 40,000 cells/μg of fibre.

A typical cellular aggregate comprises from about 50,000 to about $1x10^6$ cells or from about 100,000 to about 300,000 cells or more
25 particularly from about 150,000 to about 250,000 cells.

The number of cells and/or the amount of fibres can be varied depending on the size and density of the cellular aggregate to be formed. In this way the scaffold can be tailored to the production
30 requirements to give optimal size and fibre/cell density.

Following an appropriate incubation period the cellular aggregate can be directly implanted into a site within a subject. For example

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the cellular aggregate can be injected at or near a site in need of repair, augmentation or regeneration.

In further embodiments of the invention at least two cellular aggregates are loaded into a secondary scaffold. This secondary scaffold can be directly implanted into a site within a subject or alternatively cultured prior to implantation. The incubation of the secondary scaffold prior to implantation allows the primary cellular aggregates to fuse into a larger aggregate, which may be more stable at the time of implantation. These scaffolds are typically incubated for between 12 hours and 7 days. Furthermore the use of a secondary scaffold to contain the primary cellular aggregates provides additional mechanical strength and protection for the primary cellular aggregates during implantation and additionally while the tissue is being regenerated or repaired.

The fibres, primary and secondary scaffolds can be formed of any suitable biocompatible material. A biocompatible material is defined as having the property of being biologically compatible by not producing a toxic, injurious or immunological response in living tissue.

The fibres or scaffolds can be formed of inorganic materials selected from the group consisting of calcium phosphates, calcium carbonates, calcium sulfates or combinations thereof; organic materials selected from the group of biopolymers consisting of a collagen, gelatin, a hyaluronic acid, a proteoglycan, chitin, chitosan, chitosan derivatives, fibrin, dextran, agarose, calcium alginate, silk or combinations thereof, or synthetic polymeric materials selected from the group consisting of aliphatic polyesters, poly(amino acids), poly(propylene fumarate), copoly(ether-esters), polyorthoesters, polyalkylene oxalates, polyamides, polycarbonates, polycaprolactones, poly(iminocarbonates), polyorthoesters,

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polyoxaesters, polyamidoesters, polyoxaesters containing amine groups, polyanhydrides, polyphospazenes, polyurethanes, hydroxybutyrate, dioxanone, or hydrogels such as polyacrylates, polyvinyl alcohols, polyethylene glycols or polyethylene imines or
5 any co-polymers, blends or chemical derivatives thereof.

The aliphatic polyester can be a polylactic acid or a polyglycolic acid or copolymer or blends thereof.

10 Suitable scaffolds are formed of copolymers comprising the following monomers or mixtures of polymers and/or copolymers formed thereby: lactic acid, glycolic acid; caprolactone; hydroxybutyrate; dioxanone; orthoesters; orthocarbonates; aminocarbonates.

15 In preferred embodiments of the invention at least part of the fibres and/or primary scaffold and/or secondary scaffold is a biodegradable, bioresorbable or bioabsorbable material.

In embodiments of the invention in which the fibres and the primary
20 scaffolds are bioresorbable, it is preferable that only a residual amount of scaffold material remains after culture, with the scaffold material having been replaced by the seeded cells and the secreted matrix proteins.

25 In embodiments of the invention the at least two primary scaffolds are formed of the same material. Alternatively the primary scaffolds are formed of different materials.

In embodiments of the invention the at least two primary scaffolds
30 and the secondary scaffold are formed of the same material. Alternatively at least one of the primary scaffolds is formed of a different material to the secondary scaffold.

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In embodiments of the invention the plurality of fibres can consist of fibres made of the same material. Alternatively the plurality of fibres can consist of fibres made of the different materials.

5 In embodiments of the invention at least part of the fibres and/or primary scaffolds and/or secondary scaffolds are porous. This porosity enables cell migration and nutrient flow throughout the scaffold and prevents the interior of the scaffold becoming anoxic and therefore void of any cellular components.

10

In particular embodiments of the invention the primary scaffolds are made of non-woven felts.

The methods according to the present invention optionally comprise 15 the step of incubating the cell-seeded fibres and/or primary scaffolds and/or the secondary scaffolds in the presence of a biological agent and/or a chemical agent.

The at least one biological agent and/or a chemical agent can be 20 provided in the culture media. The cells can be exposed to the at least one biological agent and/or a chemical agent constantly or intermittently throughout the duration or the incubation period.

Alternatively the at least one biological and/or chemical agent is 25 associated with at least part of the fibres and/or primary and/or secondary scaffolds.

The at least one biological and/or chemical agent is selected from the group consisting of differentiation agents, growth factors, matrix 30 proteins, peptides, antibodies, enzymes, cytokines, viruses, nucleic acids, peptides, osteogenic factors, chondrogenic factors, immunosuppressants, analgesics or combinations thereof.

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For example, the following actives may be used in the culture of cells or additionally delivered with the scaffold implant: members of the transforming factor beta (TGFβ) family, members of the bone morphogenetic protein (BMP) protein family, members of the fibroblast growth factor (FGF) family, platelet derived growth factor, (PDGF), parathyroid hormone related peptide (PTHrP), insulin or insulin-like growth factor, bisphosphonates or pyrophosphates.

During the incubation period cells can remain undifferentiated or they can be induced to partially differentiate (ie are "primed") or completely differentiate along at least one appropriate cell lineage. This can be achieved by culturing the cells in media containing differentiation factors.

In embodiments of the invention in which the cells are induced to differentiate along the chondrogenic lineage the cells are cultured in the presence of members of the transforming growth factor beta (TGFβ) family, for example TGFβ-3

According to a fifth aspect of the invention there is provided a tissue implant obtainable by the method according to the first or third aspects of the invention.

According to a sixth aspect of the invention there is provided a kit of parts comprising at least two primary scaffolds and a second scaffold.

According to a seventh aspect of the invention there is provided a kit of parts comprising at least a plurality of fibres and a second scaffold.

The kit can further comprises a cell source, such as frozen cells.

The kits can further comprise means for isolating cells from an appropriate tissue source, such as surgical instruments for use in tissue biopsies and/or means for comminuting the tissue, for example mechanical devices and/or enzymes.

5

The kits can further comprise appropriate media and/or tissue culture vessels.

The kits can further comprise at least one biological and/or chemical
10 agent selected from the group consisting of differentiation agents, growth factors, matrix proteins, peptides, antibodies, enzymes, cytokines, viruses, nucleic acids, peptides, osteogenic factors, chondrogenic factors, immunosuppressants, analgesics or combinations thereof.

15

In embodiments of the invention the extracellular matrix producing cells are stromal cells selected from the group consisting of chondrocytes, osteoblasts, fibroblasts, adipocytes, myoblasts, pericytes, mesenchymal stem cells or any other cell type capable of
20 synthesising an extracellular matrix component, or combinations thereof.

The extracellular matrix component can be a collagen, for example type II collagen.

25

The extracellular matrix component can be a glycosaminoglycan (GAG), for example chondroitin sulphate, dermatan sulphate, keratin sulphate, heparin sulphate, heparin or hyaluronan. GAG chains may be covalently linked to a protein to form proteoglycans.

30

The isolated extracellular matrix producing cells may be obtained from donor tissue. The donor tissue may be autologous, allogeneic or xenogeneic and may be derived from any appropriate tissue,

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including cartilage, bone, skin, tendon, ligament or meniscus. Cells can be obtained from these tissues by standard mechanical methods (e.g biopsy, dissecting, comminuting) and/or enzymatic digestion (e.g collagenase, protease, etc).

5

In alternative embodiments of the invention, the isolated extracellular matrix producing cells may be derived from a source of primary cells or an established cell line.

10 In further embodiments of the invention the extracellular matrix producing cells are stem cells or progenitor cells or combinations thereof. The stem cells or progenitor cells can be derived from a fetal, embryonic or adult source. Suitable sources of these cells include, but are not limited to, bone marrow, blood and umbilical

15 cord blood.

In particular embodiments of the invention the stem cells are mesencyhmal stem cells (MSCs). MSCs are multipotent stem cells that can differentiate into a variety of cell types including osteoblasts,

20 chondrocytes, myocytes, adipocytes and beta-pancreatic islets cells. MSCs have also been shown to transdifferentiate into neuronal cells

In further embodiments of the invention the extracellular matrix producing cells can be genetically engineered to constitutively,

25 transiently or inducibly express a gene product beneficial for successful and/or improved transplantation.

The tissues to be augmented, repaired or regenerated by the methods according to the invention can include, but are not limited

30 to, connective tissue such as bone, cartilage, tendon, ligament, meniscus, muscle and adipose. However, other tissues are envisaged.

The subject in which a tissue is being augmented, regenerated or repaired can be a human or non-human animal.

According to an eigth aspect of the invention there are provided methods of forming a tissue implant, a tissue implant therein formed, kits for use in the preparation of the tissue implant and uses of the tissue implant as substantially herein described with reference to the accompanying Figures.

The present invention provides a method of seeding cells on milli-scaffolds to produce cellular aggregates without the need for cell-microcarrier aggregate formation.

This present invention further allows the production of pieces of tissue engineered cartilage in a continual process. Cell expansion and differentiation are performed in the same vessel in a continuous process without the requirement of changing culture vessels. This reduces the risk that the sterility of the cultures will be breached, reduces the risk of human error and reduces the number of man hours involved in the culture process. This invention is scalable allowing the large-scale manufacture of tissue engineered cartilage aggregates for use in tissue repair. Unlike inventions within the prior art the invention described herein allows for the treatment of a range of cartilage defect sizes by incorporation into different sized and/or compositions of secondary scaffolds without the need to engineer large pieces of cartilage with the technical difficulties that this encounters. This invention allows the production of a single continuous piece of cartilage by the fusion of smaller pieces of cartilage tissue.

The present invention provides a method of seeding cells on fibrous-scaffolds to produce cellular aggregates without the need for cell-microcarrier aggregate formation.

DESCRIPTION OF THE DRAWINGS

Figure 1: 1mm x 0.5mm primary scaffolds produced by punching cutting.

5

Figure 2: A primary scaffold seeded with ovine bone marrow stem cells and cultured for 1 week.

Figure 3: The primary scaffolds illustrated in Figure 2 stained with
10 Safranin O.

Figure 4: A plurality of primary cell-seeded scaffolds loaded into a secondary cup-shaped scaffold.

15 Figure 5: A primary scaffold seeded with adult human mesenchymal stem cells and cultured for 21 days either (a) with TGF-β3 or (b) without TGF-β3, and then stained with Safranin O.

Figure 6: A primary scaffold seeded with adult ovine chondrocytes,
20 cultured for 21 days, and then stained with Safranin O.

Figure 7: 1-3mm x 0.5mm primary fibres produced by cutting.

Figure 8: A primary fibre-scaffold seeded with human bone marrow
25 stem cells immediately after centrifugation.

Figure 9: Several fibre-scaffold pellets after 10 days of culture.

Figure 10: A primary fibre-scaffold seeded with human bone marrow
30 stem cells and cultured for 10 days and stained with Safranin O

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Figure 11: A primary fibre-scaffold seeded with ovine bone marrow stem cells and cultured for 10 days and stained with Safranin O.

Figure 12: A plurality of primary cell-seeded fibre-scaffolds loaded 5 into a secondary cup-shaped scaffold.

DETAILED DESCRIPTION OF THE INVENTION

The methods outlined below can be used for the preparation of 10 tissue implants for cartilage repair.

EXAMPLE 1: Primary scaffolds seeded with ovine bone marrow stem cells

15 Step 1: Preparation of primary scaffolds

Polyglycolic acid (PGA) non-woven felt is reinforced with poly(L-lactide-co-glycolic acid (PLLGA) by dipping the felt in a solution of PLLGA and dried. Discs of between about 0.5mm x 1mm in diameter 20 and between about 0.5 – 3mm in depth were punched out.

The discs were sterilised with a 70:20:10 solution of ethanol:acetone:water, and then incubated in a 50:50 solution of foetal calf serum (FCS) and phosphate buffered saline (PBS) for 2 25 hours at room temperature to coat the felts with FCS components that aide cell adhesion.

Step 2: Seeding cells onto primary scaffolds

30 Ovine bone marrow stem cells were seeded onto 45 primary scaffolds at cell number of about 250,000 cells/scaffold. The primary scaffolds were seeded in a falcon tube in a total volume of 5ml α MEM media containing 10% HIFCS, 2mM L-glutamine, 1% non-

essential amino acids, 100IU/ml penicillin, 100μg/ml streptomycin, 50μg/ml ascorbic acid and 5ng/ml FGF-2. Cells and scaffolds were cultured overnight on a Spiromix* platform to constantly bathe the scaffolds in the cell-containing media. After 24 hours the media
5 volume was made up to 25ml and transferred to non-adherent vented tissue culture conical flasks. The cells were cultured with constant agitation on a flat bed shaker for a further week with media being changed every 2-3 days. Figure 2 shows an example of a cell seeded scaffold which has been cultured for 1 week showing the
10 infiltration of cells in the scaffold.

Step 3: Culture of cell-seeded primary scaffolds in the presence of chondrogenic agents.

15 After 1 week the cell-seeded scaffolds were transferred to chondrogenic differentiation media consisting of low-glucose DMEM containing 2mM L-glutamine, 1% non-essential amino acids, 100IU/ml penicillin and 100μg/ml streptomycin, containing the following 1×10^{-7}M dexamethasone, 50μg/ml ascorbic acid, 1×ITS
20 (insulin, transferring, selenious acid), 40μg/ml proline, 1mM sodium pyruvate and 20ng/ml TGFβ3. Scaffolds were cultured for up to 28 days in this media in conical flasks with constant agitation on a flat bed shaker with the media being changed every 3-4 days.

25 Step 4: Staining of scaffold using Safranin O

Figure 3 illustrates that the ovine bone marrow stem cells have differentiated during the culture period into chondrocytes which have secreted an extracellular matrix comprises glycosaminoglycans.

30 *Trade-mark

Step 5: Loading of primary scaffolds into secondary scaffolds.

The cell-seeded primary scaffolds were then seeded into a cup-shaped secondary scaffold formed of non-woven PGA felt reinforced with PLLGA and further cultured for up to 7 days prior to implantation/storage. Figure 4 shows an example of a cup scaffold seeded with a 35 of primary cell-seeded scaffolds prior to culture.

EXAMPLE 2: Primary scaffolds seeded with adult human bone marrow stem cells

Step 1: Preparation of primary scaffolds

Polyglycolic acid (PGA) non-woven felt is reinforced with poly(L-lactide-co-glycolic acid (PLLGA) by dipping the felt in a solution of PLLGA and dried. Discs of between about 0.5mm x 1mm in diameter and between about 0.5 – 3mm depth were punched out.

The discs were sterilised with a 70:20:10 solution of ethanol:acetone:water, and then incubated in a 50:50 solution of FCS and PBS for 2 hours at room temperature to coat the felts with FCS components that aide cell adhesion.

Step 2: Seeding cells onto primary scaffolds

Adult human bone marrow stem cells were resurrected and grown in 2D culture until 90% confluent. The cells were detached from the flask using a trypsin (0.05% w/v) and EDTA (0.02% w/v) solution and then α-MEM media containing 10% FCS was added to the cell suspension to neutralize the activity of trypsin. The cells were counted and the volume adjusted to give a concentration of cells of 250,000 cells per 500 μl of media.

The discs prepared in step 1 were individually placed into single wells in a non-tissue culture treated 2ml deep polypropylene 96-well plate. Aliquots of the media containing 250,000 cells were added to the individual wells. The plates were centrifuged at 400g for 5 minutes to facilitate contact between the cells and the felt.

Step 3: Culture of cell-seeded primary scaffolds in the presence of a chondrogenic agent.

The cell-seeded discs were incubated in media supplemented with 20 ng/ml TGFß-3 for up to 18 days, with regular media changes.

Step 4: Staining of scaffold using Safranin O

Figure 5 illustrates the staining of the scaffolds with Safranin O after 18 days in culture. The arrows indicate different levels of staining intensity as follows:

1: very strong Safranin O staining
2: strong Safranin O staining
3: moderate Safranin O staining
4: non-specific Safranin O staining of PGA fibres
5: no staining

The high levels of Safranin O staining indicate chondrogenic differentiation and the secretion of a cartilage extracellular matrix. The scaffold has been resorbed by the chondrocytes.

EXAMPLE 3: Primary scaffolds seeded with adult ovine chondrocytes

Step 1: Preparation of primary scaffolds

Polyglycolic acid (PGA) non-woven felt is reinforced with poly(L-lactide-co-glycolic acid (PLLGA) by dipping the felt in a solution of PLLGA and dried. Discs of between about 0.5mm x 1mm in diameter and between about 0.5 – 3mm depth were punched out.

The discs were sterilised with a 70:20:10 solution of ethanol:acetone:water, and then incubated in a 50:50 solution of FCS and PBS for 2 hours at room temperature to coat the felts with FCS components that aide cell adhesion.

Step 2: Seeding cells onto primary scaffolds

Adult ovine chondrocytes were resurrected and grown in 2D culture until 90% confluent. The cells were detached from the flask using a trypsin (0.05% w/v) and EDTA (0.02% w/v) solution and then α-MEM media containing 10% FCS was added to the cell suspension to neutralize the activity of trypsin. The cells were counted and the volume adjusted to give a concentration of cells of 250,000 cells per 500 μl of media.

The discs prepared in step 1 were individually placed into single wells in a non-tissue culture treated 2ml deep polypropylene 96-well plate. Aliquots of the media containing 250,000 cells were added to the individual wells. The plates were centrifuged at 400g for 5 minutes to facilitate contact between the cells and the felt.

Step 3: Culture of cell-seeded primary scaffolds in the presence of a chondrogenic agent.

The cell-seeded discs were incubated in media supplemented with 20 ng/ml TGFβ-3 for
5 up to 18 days, with regular media changes.

Step 4: Staining of scaffold using Safranin O

Figure 6 illustrates the staining of the scaffolds with Safranin O after 18 days in culture.
10 The arrows 7 and 8 indicate different levels of staining intensity.

The high levels of Safranin O staining indicate chondrogenic differentiation and the secretion of a cartilage extracellular matrix. The scaffold has been resorbed by the chondrocytes.

15

EXAMPLE 4: The use of a plurality of fibres in the preparation of cell scaffolds

Step 1: Preparation of primary scaffolds

20 Polyglycolic acid (PGA) fibres were produced by chopping fibres into 0.5 - 3mm in lengths (FIG. 7).

The fibres were sterilised with a 70:20:10 solution of ethanol:acetone:water, and then incubated in a 50:50 solution of foetal calf serum (FCS) and phosphate buffered saline
25 (PBS) for 2 hours at room temperature to coat the felts with FCS components that aid cell adhesion.

30

Step 2: Seeding cells onto primary scaffolds

Sixty five micrograms of fibers prepared according to Step 1 were suspended in a total volume of 5ml DMEM media containing, 2mM L-glutamine, 1% non-essential amino acids, 100IU/ml penicillin, 100μg/ml streptomycin, 50μg/ml ascorbic acid, $1x10^{-7}$ M dexamethasone, 50μg/ml ascorbic acid, 1×ITS (insulin, transferrin, selenious acid), 40μg/ml proline, 1 mM sodium pyruvate and 20ng/ml TGFβ3. Cells (human and ovine bone marrow derived mesenchymal stem cells) at a concentration of 40,000 cells/μg of fibre (a total cell number of $5x\ 10^6$ in the 5 ml of fibres and media mentioned above) were added to the fibre suspension. The above suspension was then aliquoted at a volume of 500 μl per sample into individual wells of a sterile 2 ml deep polypropylene 96 well plate and centrifuged at 200g for 5 min to compact the cells and the fibres together (see FIG. 8). This produced 10 cell/fibre pellets. The empty wells surrounding the samples were filled with PBS to increase the humidity in the microenvironment surrounding the samples. The samples were then incubated at 37 °C, 5% CO_2 and 90% humidity for 10 days and resulted in the pellets shown in FIG. 9.

Step 3: Staining of cell/fibre aggregates using Safranin O

FIG. 10 illustrates that the human bone marrow derived stem cells differentiated during the culture period into chondrocytes which have secreted an extracellular matrix comprises glycosaminoglycans. Figure 11 shows similar results obtained using ovine bone marrow stem cells.

Step 5: Loading of primary fibre-scaffolds into secondary scaffolds.

The cell/fibre aggregates were seeded into a cup-shaped secondary scaffold formed of non-woven PGA felt reinforced with PLLGA and further cultured for up to 7 days prior to
5 implantation/storage. Figure 12 shows an example of a cup scaffold seeded with 6 of primary cell-seeded scaffolds prior to culture.

CLAIMS:

1. A method of preparing a tissue implant *in vitro*, said method comprising:

(a) providing cells isolated from a tissue source;

(b) seeding at least two primary scaffolds with the cells and culturing the at least two primary scaffolds for a period of time sufficient for the cells to secrete an extracellular matrix; and

(c) loading the at least two primary scaffolds obtained in (b) into a secondary scaffold whereby the secondary scaffold contains and protects the at least two primary scaffolds.

2. The method of claim 1, wherein the at least two primary scaffolds comprise a plurality of fibres.

3. The method according to claim 1 or 2, wherein the cells are stromal cells selected from the group consisting of chondrocytes, osteoblasts, fibroblasts, adipocytes, myoblasts, pericytes, mesenchymal stem cell, cell types which synthesize an extracellular matrix, and combinations thereof.

4. The method according to claim 3, wherein the extracellular matrix comprises a collagen or a glycosaminoglycan.

5. The method according to claim 1 or 2, wherein the cells are stem cells, progenitor cells, or combinations thereof.

6. The method according to claim 5, wherein the stem cells are mesencyhmal stem cells.

7. The method according to claim 5 or 6, wherein the stem cells or progenitor cells are derived from a fetal, embryonic or adult source.

8. The method according to any one of claims 1 to 7, wherein the cells are autogeneic, allogeneic or xenogeneic.

9. The method according to any one of claims 1 to 8, wherein the fibres, primary and secondary scaffolds are formed of:

inorganic materials selected from the group consisting of calcium phosphates, calcium carbonates, calcium sulfates, and combinations thereof;

organic biopolymers selected from the group consisting of collagen, gelatin, a hyaluronic acid, a proteoglycan, chitin, chitosan, chitosan derivatives, fibrin, dextran, agarose, calcium alginate, silk and combinations thereof;

synthetic polymeric materials selected from the group consisting of aliphatic polyesters, poly(amino acids), poly(propylene fumarate), copoly(ether-esters), polyorthoesters, polyalkylene oxalates, polyamides, polycarbonates, polycaprolactones, poly(iminocarbonates), polyorthoesters, polyoxaesters, polyamidoesters, polyoxaesters containing amine groups, polyanhydrides, polyphospazenes, polyurethanes, hydroxybutyrate, dioxanone, hydrogels, and co-polymers, blends and chemical derivatives thereof; or combinations thereof.

10. The method according to claim 9, wherein the hydogels are selected from the group consisting of polyacrylates, polyvinyl alcohols, polyethylene glycols, polyethylene imines, and combinations thereof.

11. The method according to claim 9, wherein the aliphatic polyester is a polylactic acid, a polyglycolic acid, a copolymer thereof, or a blend thereof.

12. The method according to any one of claims 1 to 11, wherein at least part of the fibre, primary scaffolds, and/or secondary scaffold is a biodegradable material.

13. The method according to claim 12, wherein the at least part of the fibre, primary scaffolds, and/or secondary scaffold is porous.

14. The method according to any one of claims 1 to 13, wherein the method further comprises incubating the cell-seeded fibres, primary scaffolds and/or the secondary scaffold in the presence of a biological agent and/or a chemical agent.

15. The method according to claim 14, wherein the at least one biological agent and/or chemical agent is associated with at least part of the fibre, the primary scaffolds, and/or the secondary scaffold.

16. The method according to claim 14 or 15, wherein the at least one biological agent and/or chemical agent is selected from the group consisting of differentiation agents, growth factors, matrix proteins, peptides, antibodies, enzymes, cytokines, viruses, nucleic acids, osteogenic factors, chondrogenic factors, immunosuppressants, analgesics, and combinations thereof.

17. A tissue implant obtained by the method according to any one of claims 1 to 16.

FIG. 1

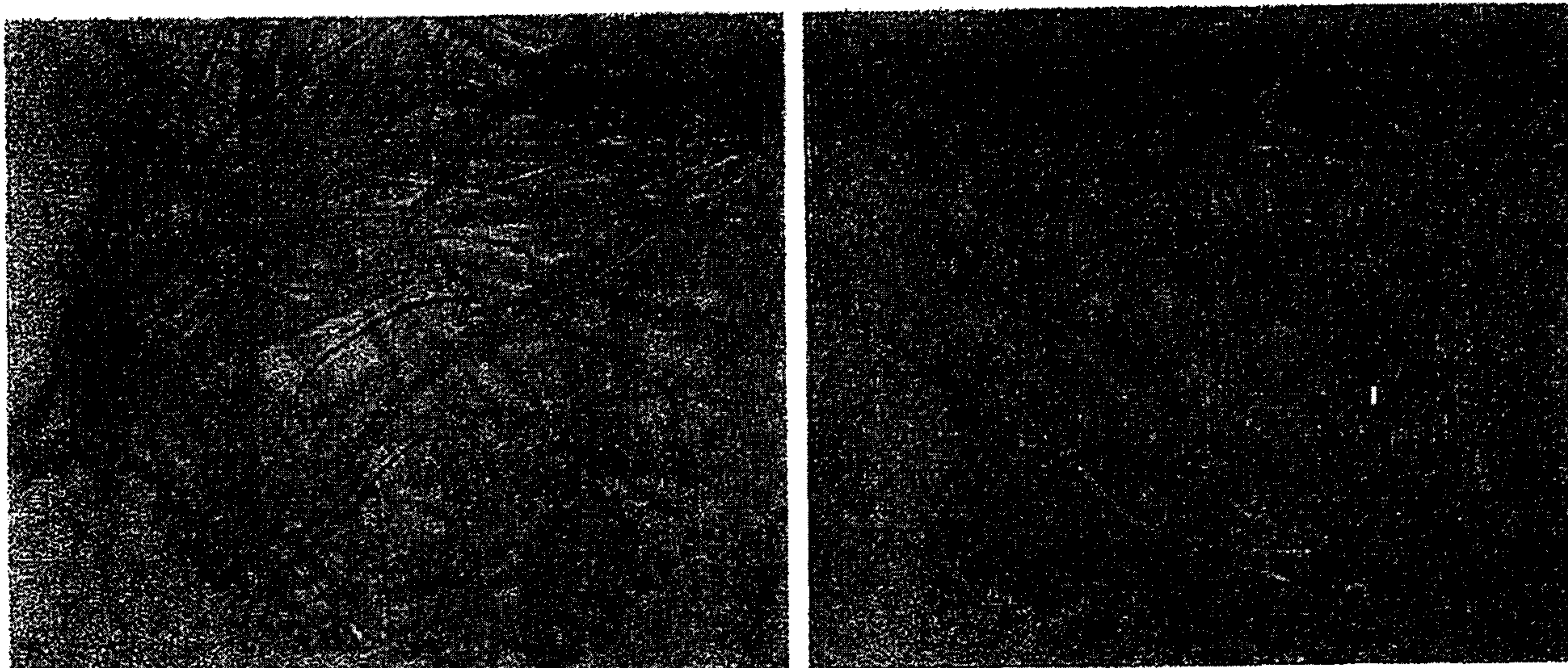


FIG. 2

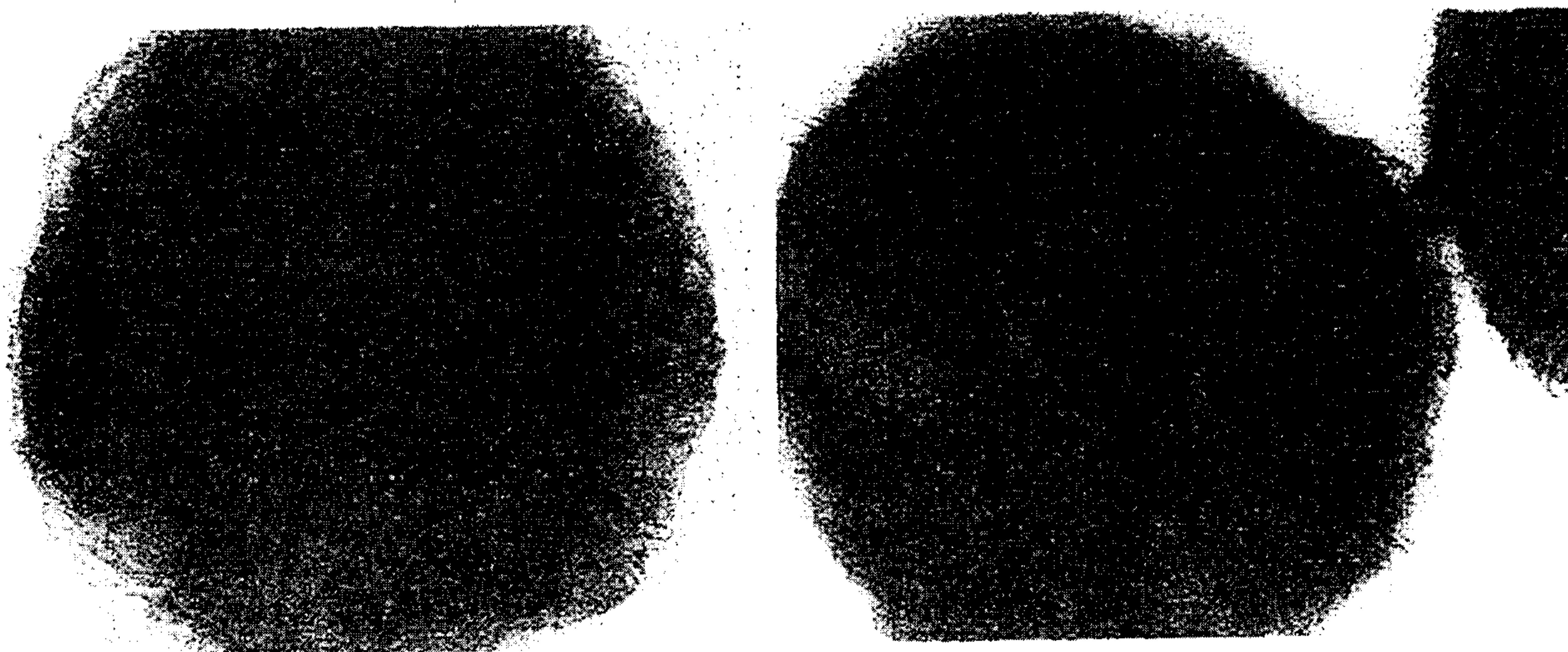


FIG. 3

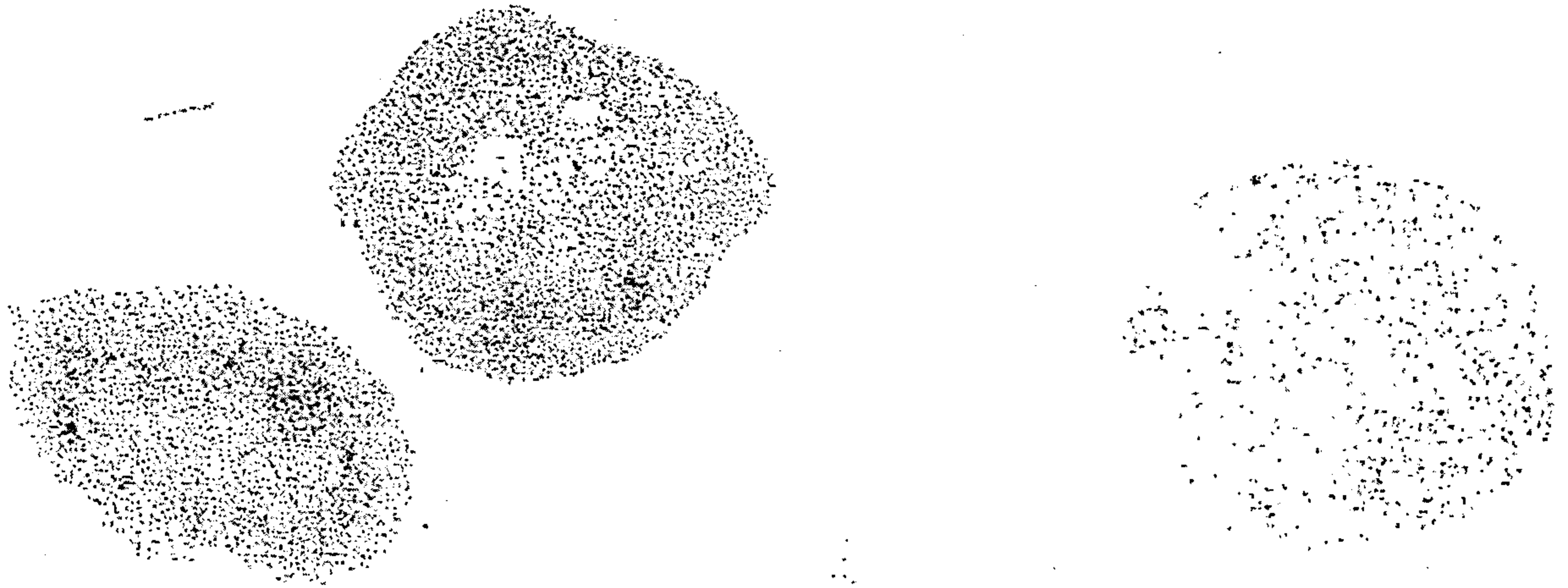
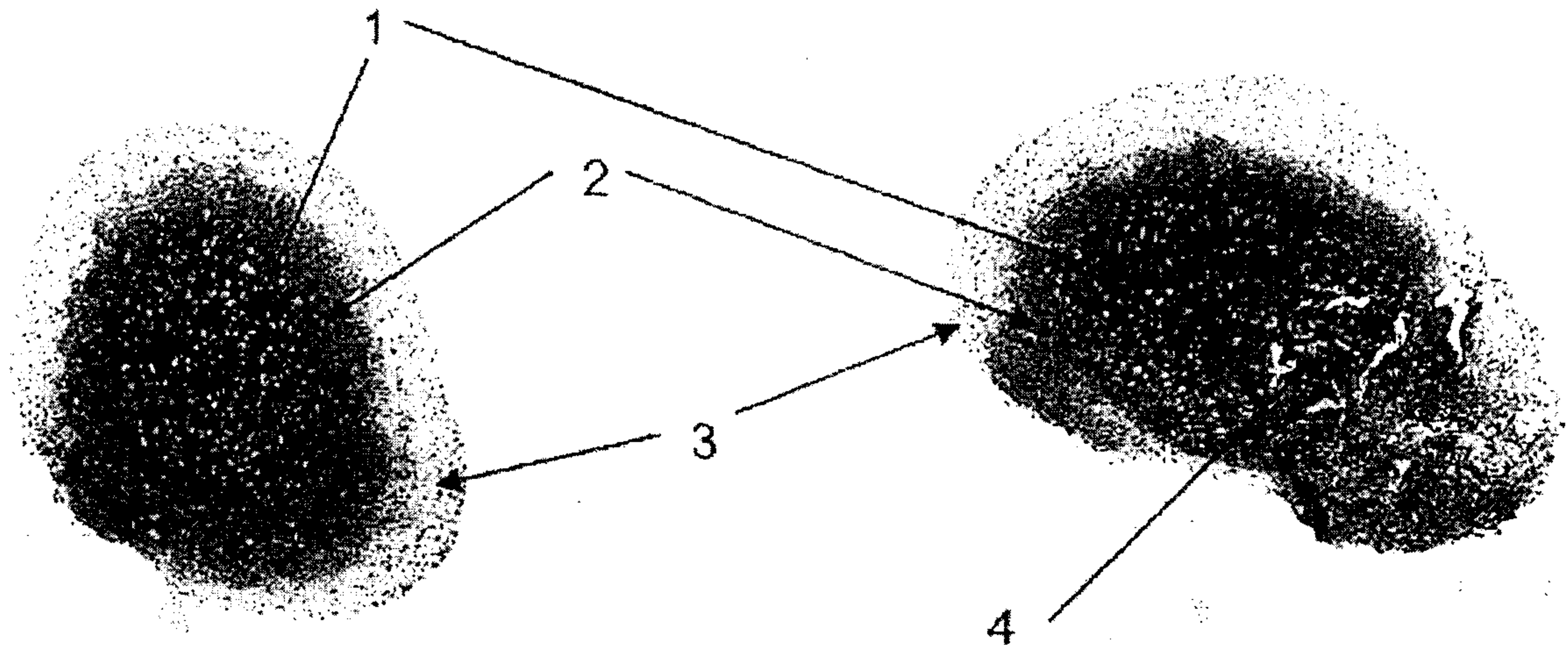


FIG. 4

FIG. 5

(a)



(b)

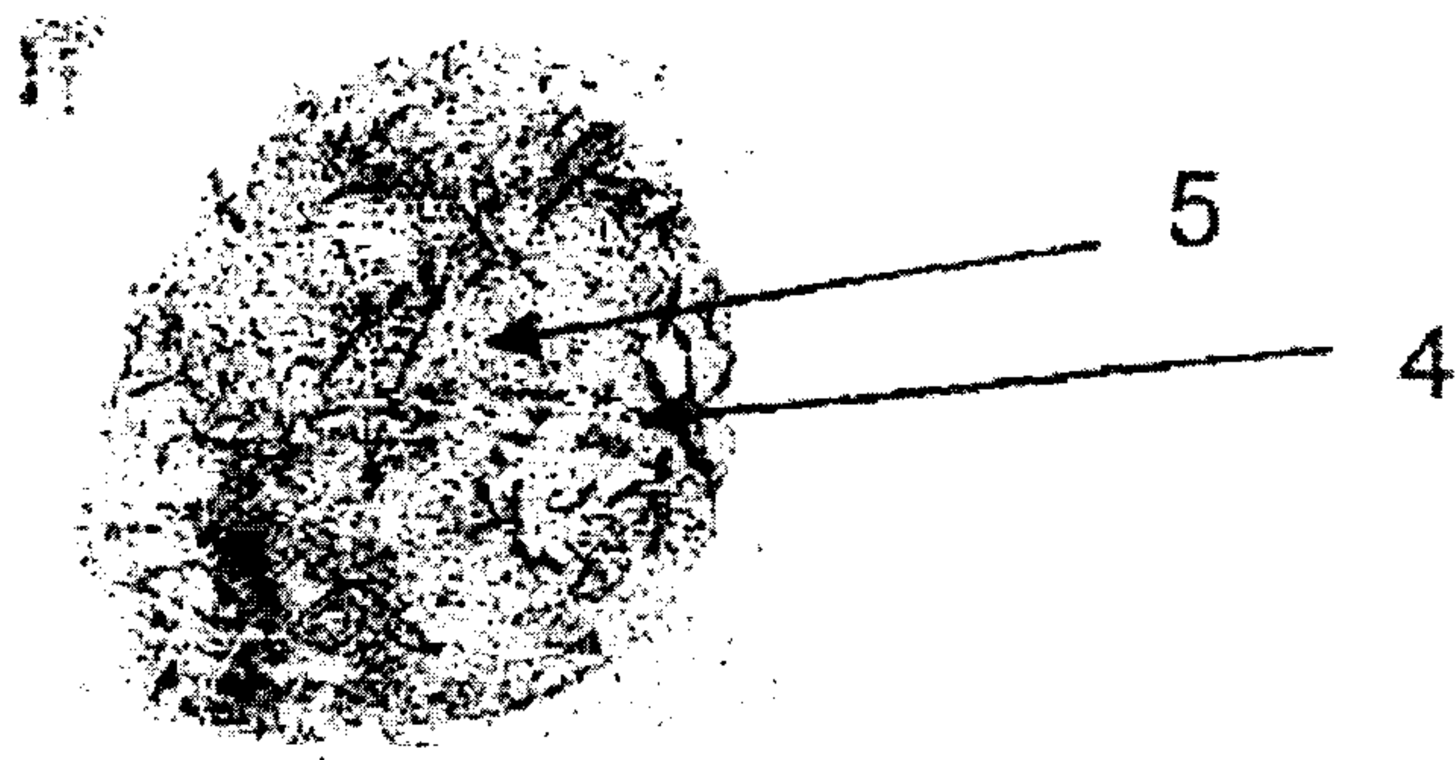


FIG. 6

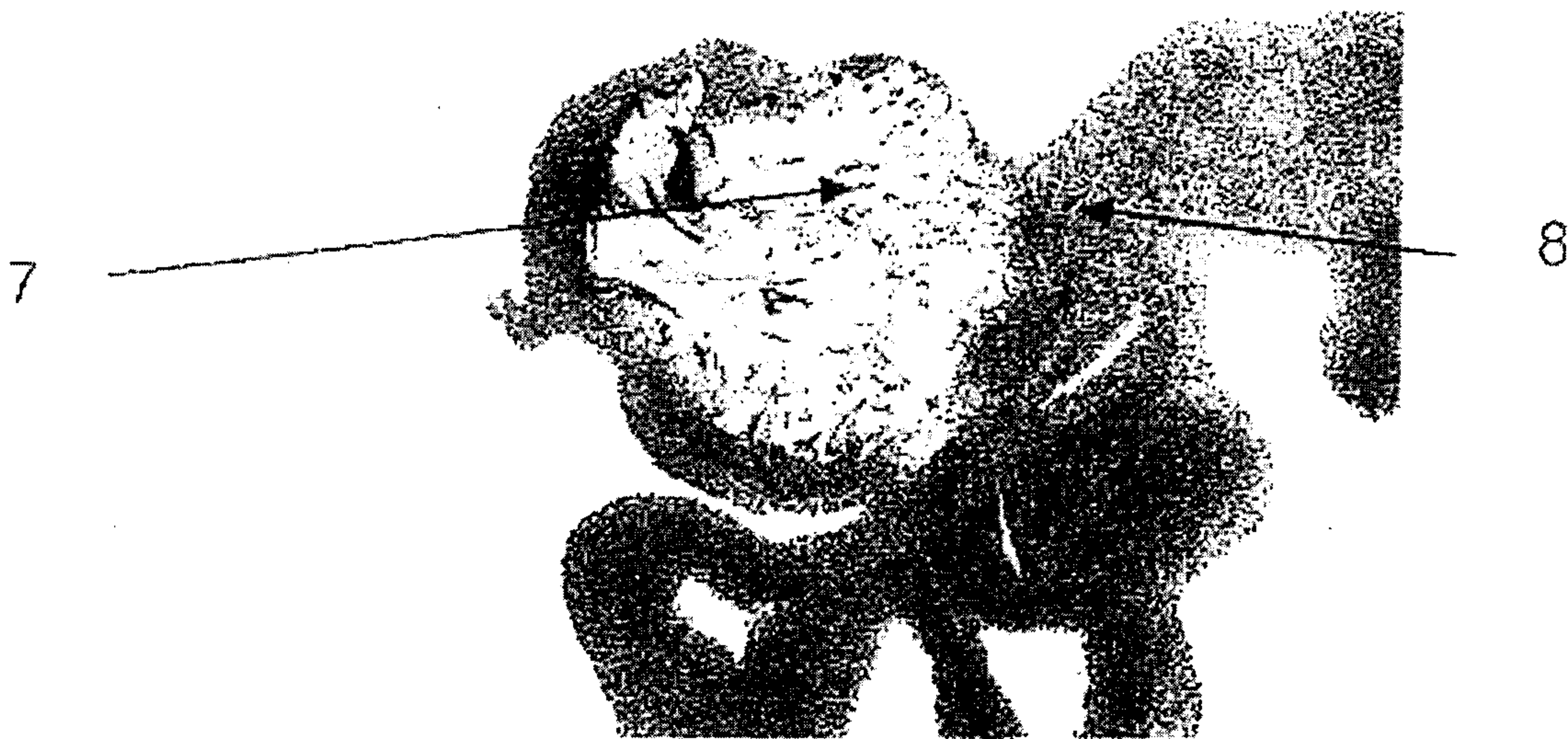


FIG. 7

FIG. 8

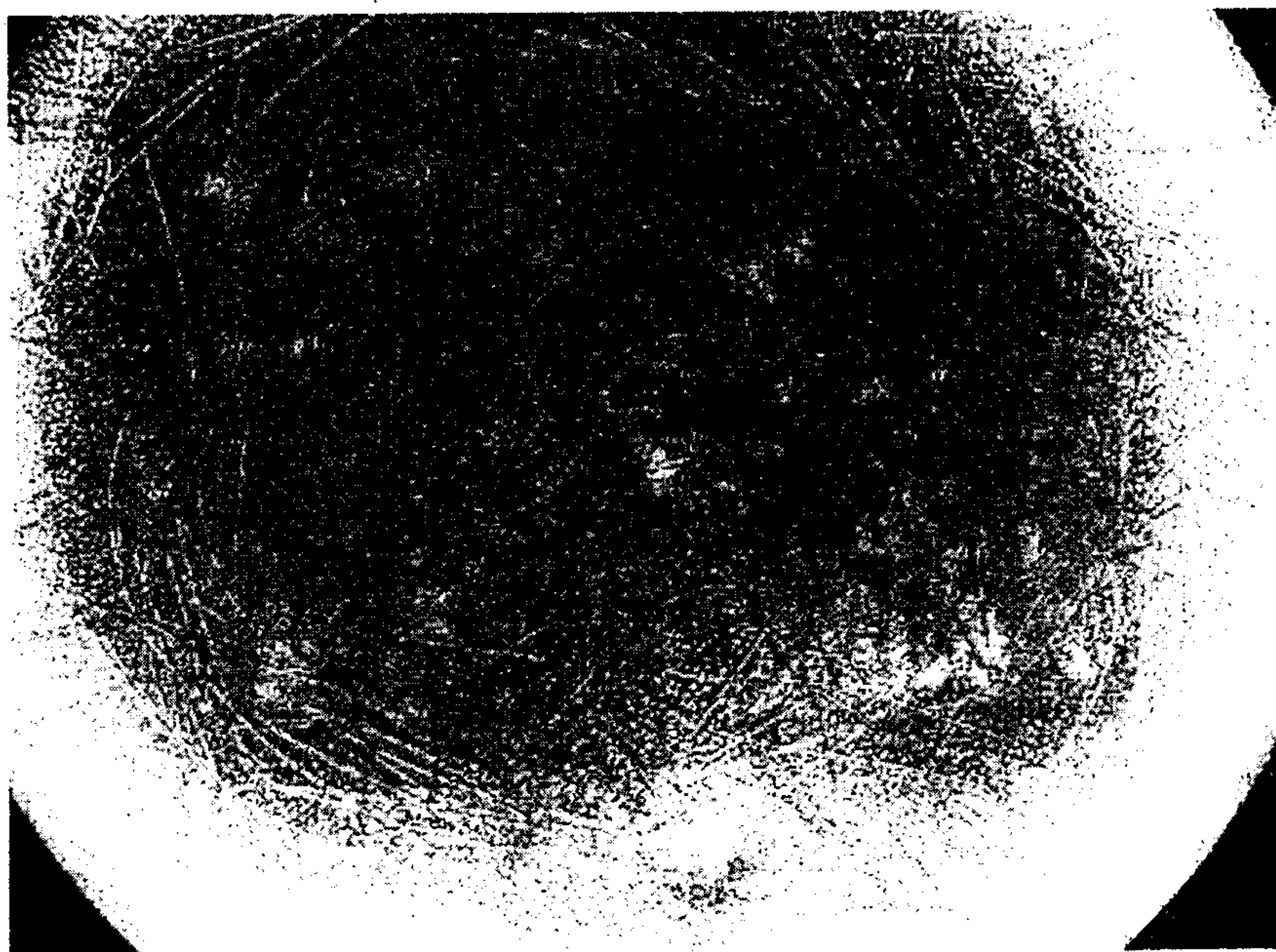


FIG. 9

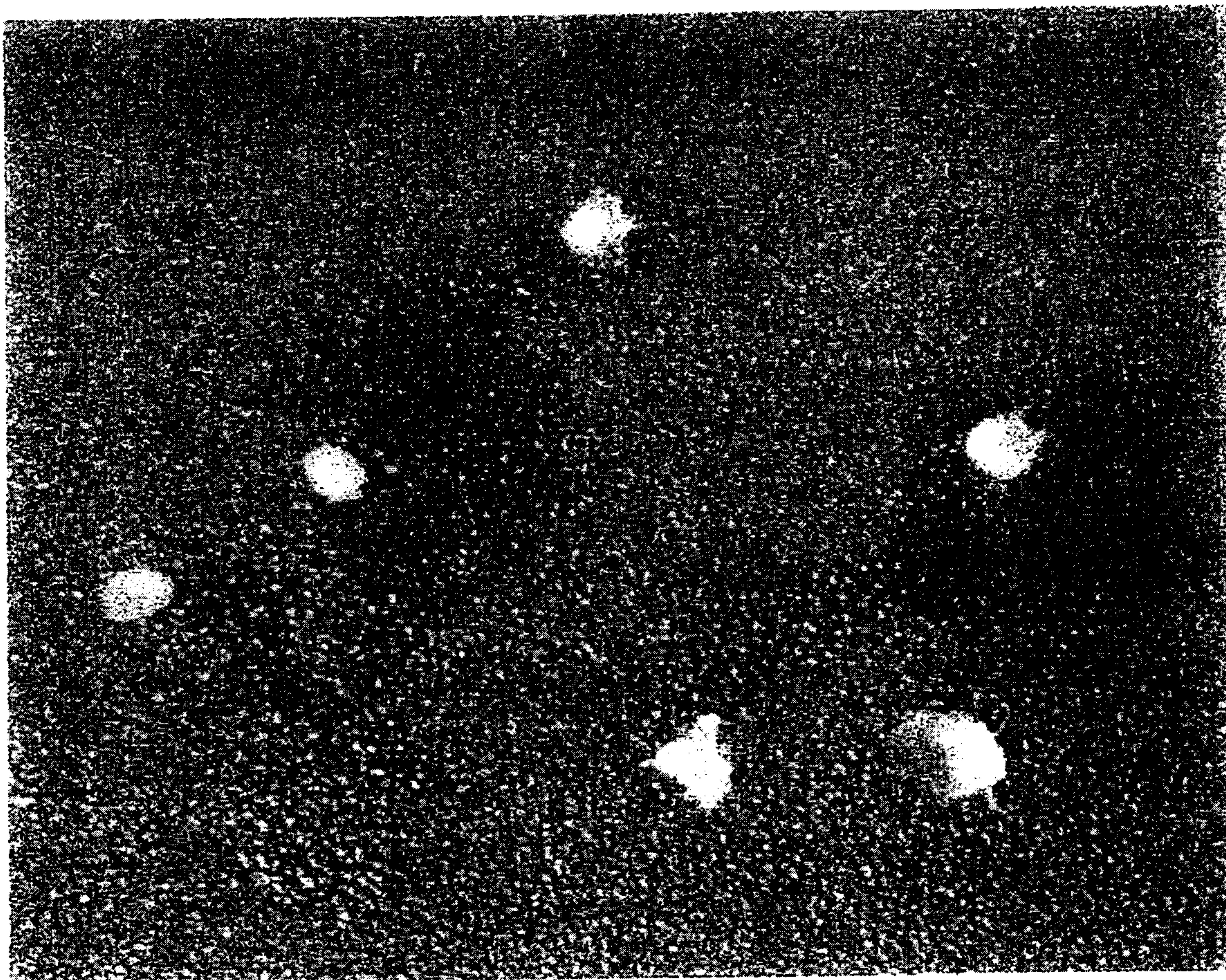
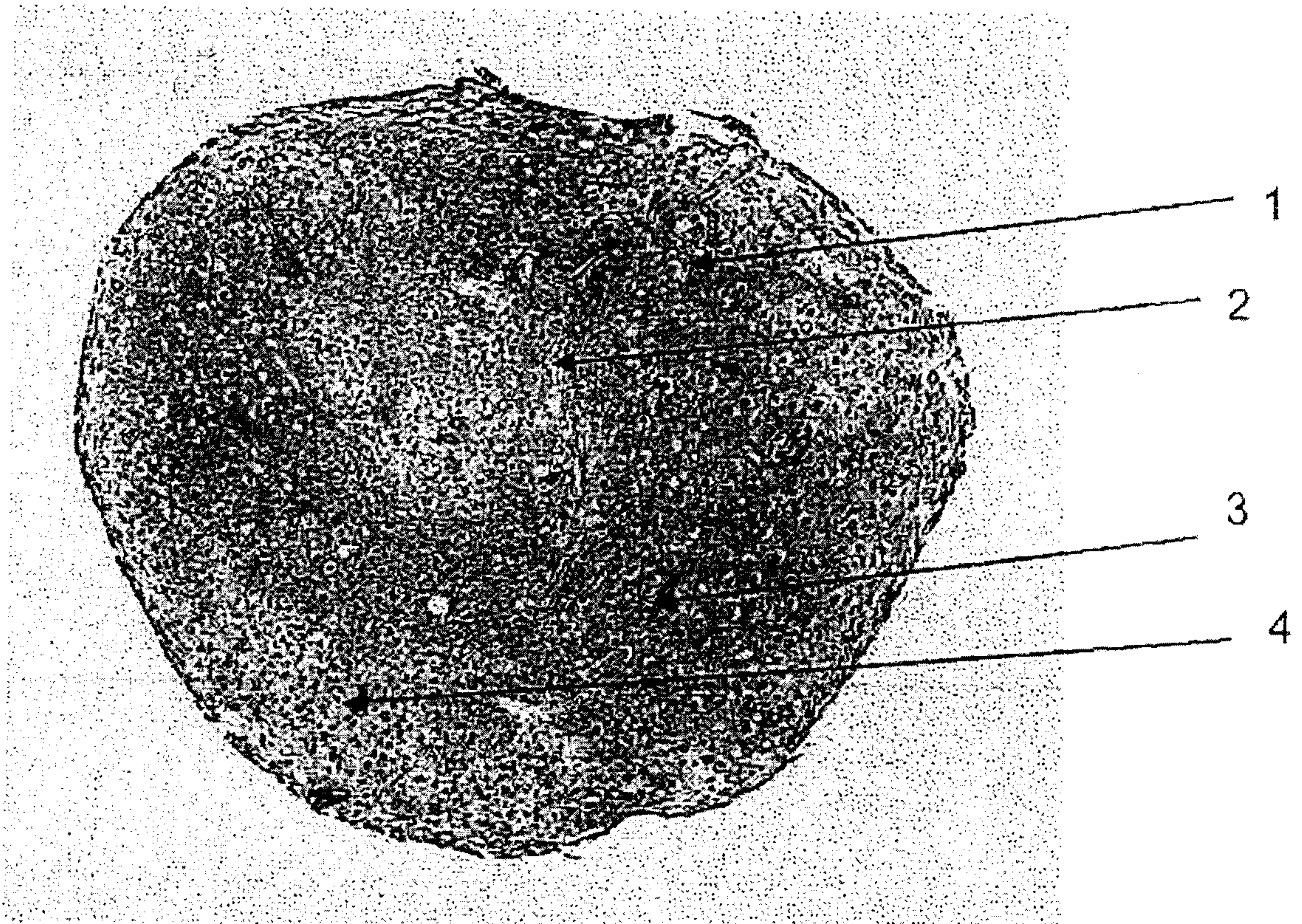


FIG. 10



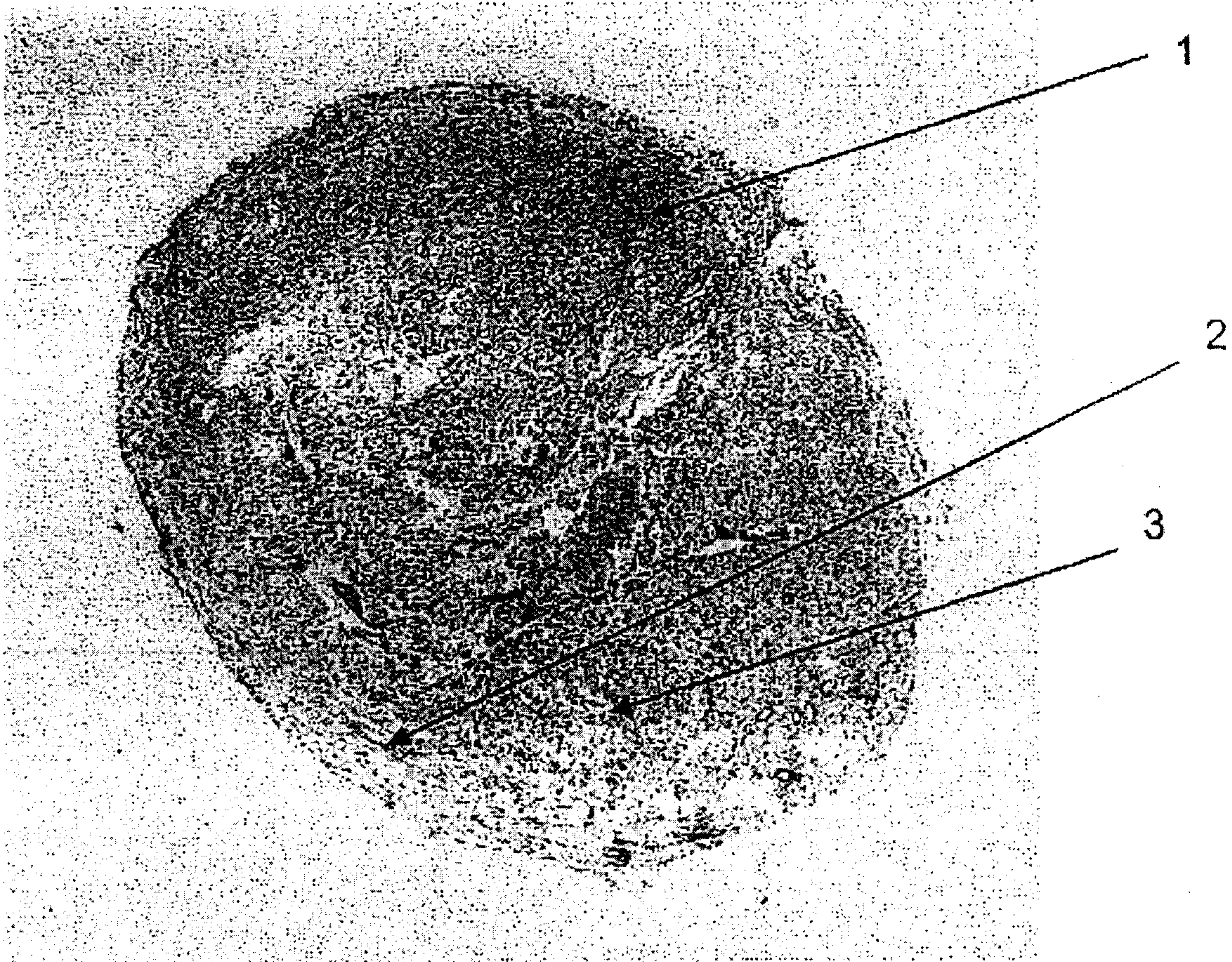
1: strong Safranin O staining

2: moderate Safranin O staining

3: non-specific Safranin O staining of PGA fibres

4: no staining

FIG. 11



1: moderate Safranin O staining

2: non-specific Safranin O staining of PGA fibres

3: no staining

FIG. 12